IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of : Roth, G. et al
Serial no. : 10/069,557
Filed : July 22, 2002
For : New Substituted Indolinones, Their Manufacture and Their Use as Medicaments
Art Unit : 1626
Examiner : Wright, Sonya N.

DECLARATION UNDER RULE 132

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Ulrike Tontsch-Grunt, solemnly state and declare as follows:

1. My technical background is as follows: I am a trained biologist having received a Masters Degree in Biology from the University of Salzburg, Austria in 1985 and a Doctoral degree in Biology from the University of Salzburg, Austria in 1989.
I engaged in post doctoral studies at the Max-Planck Institute for Psychiatry, Department of Neuroimmunology, Martinsried, Germany from Feb.1990 to Jan.1992 and at the National Institutes of Health, National Institute of Neurological Disorders and Stroke, Bethesda, Maryland, USA from July 1992 to Nov. 1993; I joined Boehringer Ingelheim Austria, in Vienna, Austria in March 1994 as a Lab Leader specializing in anti-angiogenesis research; and presently hold the position of a Lab Leader at Boehringer Ingelheim Austria, Vienna.

2. I am familiar with the subject matter of the above-noted patent application.

3. I am familiar with the Office Action dated 09/10/2003.

4. In my capacity of Lab Leader at Boehringer Ingelheim Austria, I supervised the screening and evaluation of compounds under the research division's program directed to the development of compounds active against cell proliferation and tumours.

5. In order to demonstrate activity against solid tumours for the compounds of the present invention, the biological properties of representative compounds in accordance with the invention were tested for their ability to inhibit tumour cell proliferation by the following standard procedure (e.g. Zhu J. et al., Genes and Development 12 (19), 2997-3007, 1998; Fry D.W. et al., J.Biol.Chem. 276 (20), 16617-16623, 2001).

The following three different types of tumour cells were cultivated on gelatine-coated culture dishes (0.2 % gelatine, Sigma) at 37°C, under 5 % CO_2 in a water-saturated atmosphere:

- Hela S3 cells, which are human cervix carcinoma cells, cultivated in Ham's F12 medium supplemented with 10% Foetal Calf Serum;

- NCI-H460 cells, which are human non-small cell lung carcinoma cells, cultivated in RPMI 1640 medium supplemented with 10% Foetal Calf Serum;

- SKUT-1B cells, which are human uterus leiomyosarcoma cells, cultivated in MEM medium supplemented with 10% Foetal Calf Serum, 1% non-essential amino-acids, 1%(1 mM) Na-pyruvate and 1%(2mM) L-glutamine.

All the above culture media and supplements were purchased from Life Technologies.

All the culture media contained in addition 50 μM of ß-mercaptoethanol (Fluka), standard antibiotics and 100 μg/ml of heparin (Sigma).

On the first day of the experiment, the cells were detached from the culture dishes using trypsin/EDTA and washed once in serum-containing medium.

Then, the cells were seeded out in 96 wells scintillating ``Cytostar'' microplates (Amersham Life Science) in amounts of:

- 3000 cells per well in 100 μl for Hela S3 cells;
- 3000 cells per well in 100 μl for NCI-H460 cells;
- 4000 cells per well in 100 μl for SKUT-1B cells.

For each compound to be tested, stock solutions at a concentration of 0.06, 0.2, 0.6, 2, 6 and 20 μM of the compound were prepared, by dissolution of the compound in 0.2% dimethylsulfoxide (DMSO).

After 1 day of microplate culture of the cells, the cells were treated by addition of a 100 μl aliquot of each stock solution to be tested in a well of the plate, such that the final concentration of the compound in the well was either 0.03, 0.1, 0.3, 1, 3 or 10 μM, in a medium containing a final concentration of 0.1% DMSO.

The following four representative compounds of the present invention were tested for their ability to inhibit tumour cell proliferation (The chemical name and the page Nr. of the specification where each of these compounds is disclosed is also given for information):

- Example No. 1 (page 52 of the specification)
3-(Z)-{1-[4-(piperidin-1-yl-methyl)-anilino]-1-phenyl-methylidene}-5,6-dimethoxy-2-indolinone;

- Example No. 1(3) (page 53 of the specification) 3-(Z)-(1-{4-[(N-benzyl-N-methyl-amino)-methyl]-anilino}-1-phenyl-methylidene)-5,6-dimethoxy-2-indolinone;

- Example No. 1(6) (page 53 of the specification) 3-(Z)-{1-(4-(dimethylaminomethyl)-anilino]-1-phenyl-methylidene}-5,6-dimethoxy-2-indolinone;

- Example No. 4 (page 71 of the specification) 6-amino-3-(Z)-{1-[4-(piperidin-1-yl-methyl)-anilino]-1-phenyl-methylidene}-2-indolinone.

Furthermore, the following control plates were made:

- Non-treated control (cells in culture medium supplemented with 0.1% DMSO; no treatment);
- Normal growth control (cells in culture medium; no treatment);
- Medium control (only culture medium; no cells; no treatment).

All the plates were then further incubated for 72 hours at 37°C. Then, 2 µl of [2-14-C] thymidine (1.85 MBq/ml, Amersham Life Science) was added in each well in order to determine the DNA synthesis over a further incubation of 24 hours at 37°C. After the further incubation of 24 hours at 37°C, the radioactively labelled cells were washed and the radioactivity incorporated was measured using a Wallac Performer C14 counter apparatus (Wallac 1450 MicroBeta V4.40.01). Counting was performed for 60 seconds. In order to determine the inhibitory activity of the compounds according to the invention the mean value of radioactivity of the non-treated cells (non-treated control) was subtracted from the mean value of radioactivity of the cells treated with the compounds according to the invention.

The relative cell proliferation was calculated as a percentage of the non-treated control (mean value of non-treated cells) and the concentration of active substance which inhibits the proliferation of the cells by 50 % (IC_{50}) was determined.

The following Table contains the results found.

Compound (Example No.)	IC_{50} range		
	SKUT-1B cells	NCI-H460 cells	HeLa cells
1	+++	+++	++
1(3)	++	++	++
1(6)	++	+	+
4	++	+	+

\+ 1 to 10 µM

++ 0.1 to 1 µM

+++ 0.01 to 0.1 µM

In view of their inhibitory effect on the proliferation of three types of representative tumour cells, it can be concluded that the above representative compounds are suitable for treating solid tumour diseases in which the proliferation of tumour cells plays a part.

7. The above-identified results are commensurate in scope for the claimed subject matter presently under examination, as there is no reason to expect that other compounds for the presently claimed invention would not exhibit similar activity.

The undersigned petitioner declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or

both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: December 05, 2003

Signature: U. Tontsch-Grunt

Dr. Ulrike Tontsch-Grunt